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THE EVOLUTIONARY PUZZLE OF EGG SIZE, OXYGENATION AND PARENTAL CARE IN AQUATIC ENVIRONMENTS

Ines Braga Goncalves[1, 2*], Ingrid Ahnesjö[3] and Charlotta Kvarnemo[2]

[1]Institute of Evolutionary Biology and Environmental Studies, Animal Behaviour, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

[2]Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, SE-40530 Gothenburg, Sweden.

[3]Department of Ecology and Genetics/ Animal Ecology, Uppsala University, Norbyvägen 18D, SE-75236 Uppsala, Sweden

[*]Corresponding author: ines.goncalves@ieu.uzh.ch

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Abstract

Offspring fitness generally improves with increasing egg size. Yet, eggs of most aquatic organisms are small. A common but largely untested assumption is that larger embryos require more oxygen than they can acquire through diffusion via the egg surface, constraining egg size evolution. However, we found no detrimental effects of large egg size on embryo growth and survival under hypoxic conditions. We tested this in the broad-nosed pipefish, *Syngnathus typhle*, whose males provide extensive care (nourishment, osmoregulation, oxygenation) to their young in a brood pouch on their bodies. We took advantage of this species' pronounced variation in egg size, correlating positively with female size, and tested the effect of hypoxia (40% dissolved oxygen) vs. fully-oxygenated (100%) water on embryo size and survival of large vs. small eggs after 18 days of paternal brooding. Egg size did not affect embryo survival, regardless of O_2 treatment. Whilst hypoxia affected embryo size negatively, both large and small eggs showed similar reductions in growth. Males in hypoxia ventilated more and males with large eggs swam more, but neither treatment affected their position in the water column. Overall, our results call into question the most common explanation for constrained egg size evolution in aquatic environments.

INTRODUCTION

Large juveniles commonly enjoy fitness benefits compared to smaller ones [1] and juvenile and adult size are often positively related to initial egg size [2]. Should we therefore predict evolution of ever increasing egg sizes? Following Smith and Fretwell's [3] influential model, we should not due to the trade-off between offspring size and number. Moreover, even though larger offspring show better survival and/or fitness, they do so with diminishing returns [3]. However, this is not the only cost or trade-off associated with increasing egg size in aquatic environments. Because there is much lower O_2 availability in water than in air, oxygenation can be a challenging process in aquatic environments and has commonly been argued to constrain egg size evolution [4-6]. Indeed, most fish eggs are spherical, the shape most favourable for gas exchange in low water velocities [7]. However, given a round shape, an increase in egg size results in a greater increase of volume compared to the surface area. Hence, the relative surface area, where diffusion takes place, decreases as volume increases [4, 8]. Assuming that egg volume determines embryonic oxygen (O_2) requirements and egg surface area determines the ability to acquire O_2 by diffusion, limitations in O_2 availability to the embryos may constrain egg size evolution. Yet, whilst O_2 limitations have been amply demonstrated in egg masses [9-11] they have only been assumed to affect individual embryos in response to egg size. In fact, despite theoretical support for the principle [reviewed in 8], a negative effect of egg size on embryo survival due to limited O_2 uptake is yet to be confirmed [4, 6, 12, 13].

The amount of dissolved O_2 in water affects the metabolism and development of embryos [8, 14, 15] and consequently their survival. A decrease in dissolved oxygen (DO) concentrations in the water can lead to hypoxia [i.e. any level of reduced DO that negatively affects the physiology or behaviour of an organism, 16], which can reduce embryo metabolism [17],

68 decrease yolk conversion efficiency [18], reduce development rates and delay hatching times
69 [19], as well as induce premature hatching [19], cause deformities [20], decrease size at
70 hatching and reduce post-hatching survival [19]. The hypothesis that large eggs do worse than
71 smaller ones assumes that O_2 consumption increases proportionally with egg volume [4-6].
72 However, if O_2 consumption increases at a slower rate than volume, smaller eggs can be
73 predicted to perform worse [4]. This was demonstrated in Atlantic salmon, *Salmo salar*,
74 where embryo O_2 requirements do not increase in direct proportion to the volume, suggesting
75 that at low DO concentrations, embryos of small eggs, and not of large ones, may have
76 difficulties obtaining sufficient O_2 for their development [4].
77
78 In a development of Smith and Fretwells' (1974) model, Jorgensen et al. (2011) showed that
79 optimal offspring size generally decreases with increasing growth rate. However, when the
80 authors included parental care in the model in the form of live bearing, offspring survival
81 became a function of parental survival, with larger parents predicted to produce larger
82 offspring. This prediction is in accordance with interspecific patterns among ectothermic
83 animals, which indicate that parental care and egg size are positively associated [21-23]. In
84 aquatic environments, how and whether parental care and egg size coevolved is complex to
85 disentangle, but parental care often involves the task of oxygenation of developing embryos
86 [6, 23]. In giant water bugs (Belostomatidae) selection on egg size and embryo oxygenation
87 are key factors in the evolution of paternal care [24]. Males carry the relatively large eggs on
88 their backs and promote O_2 diffusion through the egg membranes by "pumping" the brood in
89 and out of water or by brushing the eggs with their hind legs [24]. Among fishes there is
90 substantial variation in egg size [25, 26], with larger egg sizes found in demersally than in
91 pelagically spawning species [25]. Concurrently, parental care is more common in the former
92 than in the latter group [27] and a variety of parental care strategies directly improve the

93 access of O_2 to the embryos. For example, parents may choose nest sites according to O_2
94 availability [28, 29], fan their eggs to improve suboptimal O_2 conditions [30, 31], increase
95 nest opening size [32, 33] or cannibalise some of the eggs to improve the oxygenation of the
96 remaining embryos [34, 35]. In fact, parental care in aquatic species may have evolved, at
97 least partially, to protect developing embryos from low O_2 availability, which in turn could
98 have favoured the evolution of larger egg sizes [6, 36].
99
100 The aim of the current study was to investigate if egg size and O_2 availability interact as
101 expected based on the hypothesis that egg size is evolutionarily constrained by O_2-acquiring
102 ability in aquatic environments. We tested this by examining embryo development and
103 survival in the broad-nosed pipefish, *Syngnathus typhle* L., a species with substantial paternal
104 care and pronounced variation in egg size, with large females producing eggs significantly
105 larger than those of small females [37]. Together with the fact that it provides extensive
106 parental care and lives in an environment in which ambient O_2 levels vary naturally from
107 hypoxic to hyperoxic [38-41], these characteristics make it a very apt model organism. As is
108 typical for many pipefishes and seahorses (Syngnathidae), males of this genus care for the
109 developing embryos in a sealed brood pouch where they protect, osmoregulate, oxygenate and
110 transfer nutrients to the embryos through the vascularized walls of the pouch [37, 42-45].
111 Moreover, it is known from previous work on *S. typhle* that larger embryos have higher
112 respiration rates than smaller ones [46], larger eggs result in larger offspring [47] and that
113 larger juveniles enjoy higher fitness benefits [48].
114
115 We tested whether *S. typhle* embryos are negatively affected by hypoxia during development
116 and whether embryos developing from large eggs are more negatively affected by hypoxia
117 than embryos developing from small eggs. In addition, we collected behavioural data from the

118 brooding males to assess whether activity patterns and opercular movements, indicative of
119 ventilation rates, are affected by the size of the eggs they brood or the ambient O_2 saturation
120 levels they experience. The position of the brooding males was also recorded as O_2 saturation
121 levels may vary in the water column. This study provides a valuable contribution to the
122 understanding of how limited access to O_2 affects embryo development and relates to egg size
123 evolution in aquatic environments.
124
125 **METHODS AND MATERIALS**
126 *Fish collection*
127 This study was conducted at the Swedish West coast at the Sven Lovén Centre for Marine
128 Sciences, Kristineberg (58º15' N, 11º28' E). Due to time and space limitations, the study was
129 done over two consecutive summers (2007 and 2008). *S. typhle* were collected from shallow
130 eelgrass (*Zostera marina*) meadows in the vicinity of the marine station using a beam trawl
131 pulled behind a boat. For details on fish husbandry please see the Supplementary Material.
132
133 *Experimental Design*
134 Males were randomly allocated to one of four treatment groups: males brooding small or large
135 eggs in either fully oxygenated or in hypoxic water (large eggs, high oxygen (O_2): n = 12;
136 large eggs, low O_2: n = 11; small eggs, high O_2: n = 12; small eggs, low O_2: n = 12). All males
137 and females had their standard body length (SL) measured, to the nearest mm, before being
138 introduced into the mating tanks. Only medium sized males (mean SL ± SE: 170.3 ± 0.9 mm,
139 range = 155 - 181 mm, n = 47) were used to limit potential size-related differences in male
140 condition, feeding ability, quality of paternal care provided to the embryos and maternal
141 effects resulting from female choice [49]. Male SL did not differ between treatments (1-way
142 ANOVA: $F_{3,43} = 0.41$, $p = 0.75$). Since female SL is significantly and positively correlated

143 with egg size [37], females of small (range 160 – 180 mm) and large (230 – 260 mm) sizes
144 were chosen to obtain two distinct egg size categories. Males brooded on average 85 ± 25
145 eggs (mean ± SD, range 25 – 138) which did not differ between O_2 treatments (t-test: t = 0.90,
146 p = 0.37, df = 45). However, males mated with large females received 17% fewer eggs than
147 males mated with small females, and thus, brooding small eggs (t-test: t = 2.23, p = 0.03, df =
148 45, small eggs: 92 ± 25 eggs, large eggs: 77 ± 24 eggs, mean ± SD). Using the equation of the
149 relationship between female body length and egg diameter (y = 0.0026X + 1.3298) from [48],
150 to calculate egg surface areas for the two egg size treatments showed that surface area of
151 small eggs was on average 9.86 mm^2, whereas large eggs were 12.15 mm^2, resulting in total
152 egg surface areas of 907.30 mm^2 for broods of small eggs, and 935.55 mm^2 for broods of
153 large eggs. Thus, the 17% difference in number of eggs brooded by the males in the two egg
154 size treatments translated into only a 3% difference in egg surface area between treatments.
155
156 *S. typhle* is a polygynandrous species, and in our study population, males typically brood eggs
157 from 3-4 females per pregnancy [50-53]. Females produce eggs continuously [54] and large
158 females produce enough eggs to fill up almost three similar-sized males during the course of
159 one pregnancy, whilst small females can fill up little more than one male of similar size
160 during the same time period [55, 56]. Thus, males were allowed to mate polygynously, by
161 introducing groups of males in large mating aquaria (approx. 70 L) containing groups of
162 either large or small females. Specifically, females and males were introduced in the mating
163 tanks in ratios of 1:2 for large females (four females and eight males) and 1:1 for small
164 females (eight females and eight males), and individual females were replaced periodically if
165 they looked egg-limited, to ensure that males mated and filled up their brood pouches within a
166 few days. All matings took place in fully oxygenated water.
167

Each male was kept in the mating tank until his pouch was filled with eggs. Once fully mated, the male was measured and transferred into a smaller (26 x 45 x 40 cm or 26 x 35 x 35 cm) individual tank, where he was kept brooding for 18 days at a high or low level of O_2 saturation (details below). After 18 days the embryos have developed eyespots and this period corresponds approximately to between one third and one half of the total brooding period at 14-15°C [56].

Brooding aquaria were supplied with either fully oxygenated water (approx. 100% O_2, which corresponds to 8.70 mg/L or 156 mm Hg O_2 at 15°C) or hypoxic water (40% O_2, corresponding to 3.50 mg/L or 63 mm Hg O_2 at 15°C), referred to as high and low O_2 below. In natural seagrass habitats, O_2 saturation varies substantially with light and temperature, often between 60-150% [38-40, 57], but 40% is well within the natural range [40]. The hypoxic level of 40% O_2 saturation was chosen based on previous studies on sand gobies [33, 58], collected from the same bay as our pipefish, where 30-40% O_2 was used. These levels elicited behavioural changes without causing mortality.

DO concentration was decreased by pumping nitrogen into the water with the use of a MiniModule 1.7* 5.5 Membrane Contactor (Liqui-Cel, Celgard, Inc, North Carolina, USA), which removes the O_2 through a counter-current system (i.e. water and nitrogen flow in opposite directions). This unit allowed hypoxic aquaria to have flow-through water like the fully-oxygenated aquaria (see Supplementary Material for details on maintenance of O_2 treatments).

At day 18 of brooding, males were euthanized by immersion in 1ml 2-phenoxyethanol/L seawater) solution for 10 minutes, followed by severing the spinal column immediately

193 posterior to the opercula. Each male was preserved in 70% ethanol for later dissection. One
194 male was removed from all analyses because its eggs were all unfertilized.
195
196 *Embryo survival and size*
197 Males were dissected to assess relative embryo survival. This was accomplished by
198 calculating the proportion of well-developing embryos out of all eggs in the brood pouch,
199 including unfertilised eggs and substantially underdeveloped embryos.
200
201 To obtain estimates of embryo size for each brood at the end of the 18-day brooding period,
202 the average length and weight of embryos were collected as follows: Fifteen embryos, from
203 separate regions of the brood pouch, were removed from each male. The embryos were
204 separated from the egg membrane and the yolk sac. Photographs were taken of five of the
205 embryos using a camera (Leica DFC420 A) attached to a stereo microscope (Leica MZ16 A).
206 The total length (tip of rostrum to tip of tail) of each embryo (± 0.01mm) was measured from
207 the photographs of the five embryos using Leica Application Suite, version 2.7.0.RI (Build:
208 1294). The remaining ten embryos were placed on a Petri dish in a heating cupboard (60°C)
209 for one week after which the dried embryos were weighed twice on a Sartorius LE26P
210 microbalance (± 2µg). The average of these measurements divided by 10 gave the average
211 embryo weight for each male.
212
213 *Male behaviour*
214 On days 1, 9 and 18 of the brooding period ventilation rates and 10-minute video recordings
215 were taken for each male. Ventilation rates were counted during direct observations as
216 number of opercular movements of each individual for 30 seconds. Videos were analysed
217 using JWatcher v1.0. From the video-recordings, proportion of time spent above the mid-line

of the water column and proportion of time spent actively swimming were recorded. Six males were removed from these analyses because we did not have data on all days for these males.

Statistical Analysis

SPSS 22 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and PERMANOVA+ for PRIMER v6 (PRIMER-E, Plymouth, UK) were used to perform all analyses. PERMANOVA+ was chosen because its tests rely on permutations to calculate the distribution of the data, thus avoiding the strict assumptions of parametric tests that are difficult to meet when analysing groups with unequal sample sizes. Permanova has two assumptions: multivariate observations are independent (which they are between males) and identically distributed, i.e., the dispersion clouds are homogenous. To meet the second assumption, some of the data were first transformed, as detailed below.

Because some of the response variables measured were not independent from each other, e.g., embryo length, dry weight and survival, we chose to analyse our data using multivariate statistics to assess overall effects of our treatments and their interactions on our response variables. Only if the multivariate tests returned a significant effect did we then analysed the response variables separately. Permanova was used to assess: a) the effects of year (random factor (RF)), egg size treatment (fixed factor (FF): small and large) and O_2 treatment (FF: high and low), on embryo survival, length and dry weight, and b) the effects of year (RF), day (FF, repeated-measure: days 1, 9 and 18), O_2 treatment (FF), egg size treatment (FF) and male ID (RF, nested within year, egg size treatment and O_2 treatment) on ventilation rates, proportion of time spent swimming and proportion of time spent in the upper part of the water column. In (a), embryo survival was arcsine-transformed and embryo length was log-

transformed and in (b), proportion of time spent swimming and proportion of time spent in the upper half of the aquarium were arcsine-square root-transformed. Response variables were normalized (each variable had its mean subtracted and was divided by its standard deviation) in order to achieve a common scale between the variables before producing a resemblance matrix based on Euclidean distances [59, 60] for each multivariate test. As a rule, interaction terms with p (perm) > 0.2 were sequentially removed from the models, starting with the highest level of interactions, followed by within-level least significance. Due to its central importance to the questions addressed in this study, the "egg size x oxygen" interaction term was kept in the analyses of embryo survival, length and dry weight. Final models are presented in Tables 1 and 2. Whenever the multivariate permutational analyses showed significant terms, permutational ANOVA's were performed on the separate response variables, using the same reduced models and transformed variables as in the MANOVA's. The following options were chosen for all analyses: type III sums of squares, fixed effects sum to zero, model: permutation of residuals under a reduced model, number of permutations: 9999.

Since all matings were done prior to the experimental O_2 treatments the effect of O_2 treatment and of egg size treatment on number of eggs received by the males were analysed separately with t-tests. Significance level for all tests, except for the interactions mentioned above, was set at $p < 0.05$.

RESULTS

Embryo survival and size

Embryo survival was on average 75% (range: 18–97%) in all males combined. There was a significant overall variability between years in embryo survival and size (Table 1a). The

multivariate tests showed strong effects of egg size and O_2 treatments but not of their interaction (Table 1a). Post-hoc tests show that these effects were due to low O_2 level having a strong negative effect both on embryo length (Figure 1a) and on embryo weight (Figure 1b), but not on embryo survival (Table 1b). Egg size showed a strong positive effect on embryo weight and tended to affect embryo length positively, but neither egg size nor O_2 treatment affected embryo survival (Table 1b). If large eggs had done worse in low O_2, significant interactions would be expected between egg size and O_2 level however, no such interactions were found (Table 1a and 1b).

Male behaviour

There was a significant year by day interaction in the overall behaviours, but behaviours did not change significantly with day in the brooding cycle (Table 2a). Both O_2 and egg size treatments affected overall male behaviour significantly (Table 2a). Individuals differed significantly in their behaviour (Table 2a), particularly in their ventilation rates (Table 2b). Ventilation rates were significantly higher in males kept in low O_2 conditions compared to males kept in high O_2 conditions (Figure 2, Table 2b). Males brooding larger eggs swam significantly more than males brooding smaller eggs, but neither egg size nor O_2 level affected the time they spent in the upper part of the aquaria (Table 2b).

DISCUSSION

Egg size

We found no interactions between egg size and O_2 level on embryo survival or growth, despite the large intraspecific variation in egg size observed in this species [37]. This means that embryos from large eggs did not develop significantly worse than embryos from small eggs in hypoxic conditions, as has been predicted from theory [4, 6, 13]. If embryos from

large eggs have lower metabolic rates per egg volume compared to embryos from small eggs, in similarity to the scaling effect observed in brown trout, *Salmo trutta*, [4], such differences in metabolic rates may be large enough to compensate for the less favourable surface area to volume ratio of large eggs. Empirical support for this premise is mixed. For instance, in the mouth brooding cichlid, *Pseudocrenilabrus multicolour victoriae*, females from populations that experience hypoxic conditions year-round produce smaller yet more numerous eggs than females from populations that consistently experience high O_2 saturation levels [15]. However, a complementary laboratory study showed that F1 females from both populations produced larger eggs in hypoxic than in fully-oxygenated water [15]. This result lends support to the study by Einum and colleagues [4], which showed that brown trout embryos from small eggs had lower survival in hypoxic water compared to embryos from larger eggs. Together these studies have generated some controversy as to which egg size is more negatively affected by hypoxia.

In *S. typhle*, embryo respiration increases with increasing embryo dry mass with a slope of 0.44 [61]. Although the relationship between respiration and egg volume is not exactly known, we know that egg diameter and dry mass are strongly and positively correlated [37]. Thus, this relatively low slope (clearly <1) indicates that embryos from larger eggs have lower metabolic rates per egg volume compared to embryos from smaller eggs. If so, such difference in metabolic rates could explain why embryos from the small and large egg treatments were similarly affected by our hypoxic treatment. Thus, our study adds to the list of studies questioning whether embryos from larger eggs are indeed more O_2-constrained in aquatic environments.

317 Our males displayed low swimming activity, consistent with the generally inactive and cryptic
318 behaviour of syngnathids and with previous studies showing that pregnant males swim less
319 than females and non-pregnant males [62, 63]. However, males brooding larger eggs swam
320 significantly more than males brooding smaller eggs, though they did not differ in the time
321 spent in the upper part of the aquaria. This result is in line with the increased swimming
322 activity found in other species, shown to increase the amount of water that passes through the
323 gills, improving O_2 uptake [64, 65]. It also resembles the typical parental behaviour of the
324 giant waterbug, in which fathers improve the access of O_2 to the developing embryos [66]. It
325 is thus possible that swimming in *S. typhle* has similar effects, with the movements of the
326 paternal body facilitating O_2 uptake in the gills and this way improving gas exchange to meet
327 the higher O_2 requirements of larger embryos.
328
329 Males that brooded large eggs received 17% fewer eggs than males brooding small eggs.
330 Thus, our data provide a possibly unique example, in which males that mate with large
331 females pay a cost by caring for fewer offspring. Since large females are preferred as mates in
332 this species [61], this clearly suggests that the fitness benefits males gain from that preference
333 [e.g. larger offspring that survive better; 48, 67] are large enough to override the number's
334 cost. Moreover, caring for fewer embryos, i.e. having a lower embryo density in the pouch,
335 may allow for better oxygenation. Interestingly, O_2 levels in the bottom section of the pouch
336 tend to be higher compared to other sections [68] and embryo density is often lower in this
337 part of the pouch (personal observation). However, whether egg size affects O_2 levels in the
338 pouch still remains to be tested.
339
340 *Oxygenation*

The males brooding in hypoxia showed significantly faster opercular movements throughout the experiment. Faster opercular movements result in faster O_2 extraction from the water, increasing O_2 availability for the males' own metabolic needs and, supposedly, for the developing embryos. In species where parents fan the clutches to ensure a steady access of O_2 to the developing embryos, similar increases in ventilation rates with decreasing O_2 availability have been reported [e.g., 31, 33]. Yet, in our study, embryos brooded in low O_2 conditions were shorter and lighter than embryos brooded in normal O_2 conditions, indicating a clear negative effect of hypoxia on embryo development. A previous study that measured O_2 concentrations in the pouch fluid of brooding *S. typhle* kept in normoxia and hypoxia reported that pouch fluid O_2 concentrations were much lower than those of the water surrounding the males in both treatments, and 2) pouch fluid O_2 concentrations were significantly lower in the males kept in hypoxia compared to the males kept in normoxia [68]. Two important conclusions can be drawn from that study: first, it is noteworthy that pouch brooded embryos naturally develop in much poorer O_2 conditions than those of the ambient water (about 40% lower O_2); and second, that males appear to have limited capacity to buffer the developing embryos from prolonged environmental hypoxia.

Differences in length and weight of embryos brooded in high and low O_2 conditions can arise from several direct and indirect mechanisms. First, under hypoxia, tissue differentiation rates retard development, so that embryos from males kept in low O_2 conditions may be at earlier stages of development resulting in smaller sizes [69, 70]. Second, development may progress at similar rates but embryos end up smaller due to less efficient anaerobic metabolic processes [71, 72]. Thirdly, quality of parental care (for instance, nutrients provided) may differ in normal and low O_2 conditions due to the physiological stress imposed by hypoxia on the caring parent, affecting the developing young indirectly. Both developmental retardation and

366 smaller sizes at emergence under hypoxia have been recorded in a copepod [Acartia tonsa, 73]
367 showing that these mechanisms are not mutually exclusive.
368
369 Whether due to anthropogenic effects or natural causes, hypoxia affects aquatic environments
370 worldwide and in particular areas that are important for reproduction to many aquatic
371 organisms, such as estuaries, semi-enclosed areas and shallow coastal regions [74-76]. In this
372 study, we kept hypoxic conditions and temperature constant for the duration of 18 days,
373 representing a chronic exposure [> 4 days; 16]. In our study population, at the onset of the
374 reproductive season, individuals migrate into shallow protected bays with eelgrass, where
375 algal overgrowth is common [77] so that the water may become hyperoxic during the day due
376 to photosynthesis, but hypoxic at night due to algal and plant respiration [38-40, 57]. Thus,
377 this population may experience hypoxic events that are high in frequency and magnitude but
378 periodical in duration, conditions that are difficult to replicate in the laboratory. In the
379 paternal egg-guarding Plainfin Midshipman fish, *Porichthys notatus*, a species that also
380 reproduces in near-shore environments with fluctuating O_2 levels, caring males are able to
381 withstand hypoxia for twice as long as females [78]. Whether *S. typhle* males and females
382 differ in their ability to tolerate hypoxic conditions, related to the sexual difference in parental
383 care, is unknown but worth exploring in the future.
384
385 *Paternal care*
386 Brooding males provide nutrients, osmoregulation and oxygenation to the developing
387 embryos. In addition, inside the brood pouch embryos are safe from external predation so that
388 whole brood mortality depends on paternal survival, whereas individual embryo survival may
389 depend on paternal brooding abilities (nutrients, O_2 etc.) or embryo competition [45, 79]. In
390 the current study, relative embryo survival was around 75% in both treatments; a number

391 comparable to previous studies on this population [48, 67], and importantly embryo survival
392 did not differ between egg size or O_2 treatments. Does this mean males are able to adjust O_2
393 provision to the embryos in relation to embryonic consumption? Probably not significantly:
394 while we do show that males brooding large eggs swim more than males brooding small eggs,
395 which may facilitate paternal O_2 uptake and allow them to provide relatively more O_2 to
396 larger embryos, we also know that pouch fluid O_2 is considerably lower than that of the tank
397 water [68], both under hypoxia and normoxia. In addition, pouch O_2 drops markedly with
398 time in the brooding period, that is, when O_2 consumption by the embryos is likely to be
399 higher [68]. Therefore, if males are able to adjust O_2 provisioning to the embryos, such ability
400 appears to be limited .
401
402 An alternative explanation for the lack of difference in embryo survival between broods
403 consisting of the large and small egg size, and the lack of interaction between egg size and O_2
404 levels, is that with smaller egg size, more eggs fit in the pouches. Thus, if there is a benefit of
405 being a small egg in terms of oxygenation, the larger number of embryos developing in close
406 contact, which result in similar total egg surface areas in the two egg size treatments, may
407 mask it. This could be tested in future studies, by keeping egg numbers rather than pouch
408 fullness constant, while varying egg size. However, we chose not to do this in the current
409 study, because if e.g. 100 large eggs fill a male's pouch, then 100 small eggs will leave it
410 partially empty, this would have led to differences in pouch fluid volumes, and potentially
411 also the amount of O_2 available in the fluid.
412
413 Why was embryo survival not affected by hypoxia in our study, when it has been documented
414 in other aquatic species [e.g. 80, 81]? Our chosen level of 40% O_2 saturation (3.50 mg O_2/L)
415 may not have been low enough to affect embryo survival, even though it clearly affected

416 embryo size negatively and caused a significant increase in ventilation rates of brooding
417 males. A range of O_2 concentrations have been used in other studies and species showing
418 significant variation in embryo sensitivity to hypoxia [4, 81]. Yet, given that our chosen level
419 of 40% is comparatively low for natural seagrass meadows, and the exposure in our
420 experiment was considerably longer than what is common in nature, this result shows that *S.*
421 *typhle* is able to cope with brooding also at reduced O_2 levels.
422
423 Broadly, our study raises the question as to whether pouch brooding in syngnathids evolved at
424 least partly as an adaptation to hypoxia. It is common in fish for males that provide parental
425 care during embryonic development to adjust their fanning behaviour in response to ambient
426 O_2 conditions [33, 82] as well as to the developmental stage of the embryos [83]. In the
427 seahorse*, Hippocampus zosterae*, males increase O_2 consumption during brooding by up to
428 50% compared to when not brooding [84]. Interestingly, in our study, day in the brooding
429 period (i.e. embryonic stage) did not influence male ventilation rates or activity patterns.
430 However, since embryonic metabolic demands tend to peak just before hatching [8, 46, 69,
431 85-87] it is possible that hypoxia impacts embryo survival only towards the end of the
432 brooding period.
433
434 *The aquatic puzzle: egg size and oxygen under paternal care*
435 Pipefishes and seahorses display substantial variation among genera in where and how brood
436 care is performed [37, 43]. Species with embryos attached to the surface of the male body
437 (without a pouch) tend to have smaller eggs than species with enclosed pouches [37]. In the
438 absence of a pouch embryos access O_2 directly from the ambient water, whereas in pouch
439 brooders embryos depend on paternal oxygenation. In *S. typhle*, egg size is strongly correlated
440 with female body length, with large females producing eggs that are substantially larger than

441 those of small females [37]. Despite this pronounced intraspecific variation in egg size and the
442 knowledge that large embryos respire more than smaller ones [46], we found no support for
443 the hypothesis that large eggs do worse than small eggs in hypoxia due to their lower surface
444 area to volume ratio. We found that under hypoxia male ventilation rates increased
445 significantly and that embryo length and weight were significantly and negatively affected.
446 However, these effects were similar in both egg size classes and embryo survival at 18 days of
447 age did not differ in relation to ambient O_2 levels. Thus, in contrast with long-established
448 theory, embryos from large eggs were not more constrained in their development than
449 embryos from smaller eggs in males brooding either in hypoxia or normoxia. This may be due
450 to embryonic metabolic demands not increasing proportionally with egg volume, as discussed
451 above, or to the pregnancy in *S. typhle*, where males are able to provide nutrients and O_2 and
452 to osmoregulate the embryos during brooding. In the theoretical model developed by
453 Jorgensen and colleagues (2012), ecological factors favouring the evolution of large offspring
454 size also favour live bearing. In accordance with this, in *S. typhle* we find females producing
455 large eggs for their body size [37] and males providing long and multifaceted paternal care in
456 the safety of the brood pouch.
457
458 In conclusion, our study did not provide support for the surface-to-volume ratio argument for
459 constraints in egg size evolution of aquatic organisms because, although *S. typhle* produces
460 relatively large eggs and has limited ability to oxygenate the developing embryos, the
461 observed negative effects of O_2 limitation on embryo development were largely independent
462 of egg size. Thus, this hypothesised constraint is unlikely to be universal and the controversy
463 around this issue is far from settled. Our study thus highlights the need for more intra- and
464 interspecific empirical tests, on a greater variety of aquatic organisms, to better understand the
465 evolutionary puzzle of egg size, oxygenation and parental care in aquatic environments.

Author contributions:

Conceived and designed the experiments: I.B.G., I.A. and C.K. Performed the experiments: I.B.G. Analysed the data: I.B.G. Wrote the paper: I.B.G., I.A. and C.K.

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Ethics statement:

The experiment was done according to Swedish law, with an ethical approval given by the Swedish Animal Welfare Agency (permits nr 196-2005 and 112-2007). This study does not involve any endangered or protected species.

Data accessibility:

All data will be made accessible on Dryad Digital Repository.

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719

720 Table 1 – Permutational MANOVA and ANOVA analyses of the effects of year (2007, 2008),
721 egg size treatment (small vs. large) and oxygen treatment (100% vs. 40%) on *Syngnathus*
722 *typhle*, a) overall and b) separately for relative embryo survival, average embryo length (mm)
723 and dry weight (mg). N = 46. Analyses were performed on transformed and normalised
724 variables.
725

a) Multivariate test:

Source	df	MS	Pseudo-F	p (perm)
Year	1	8.92	3.80	0.018 [y]
Egg size	1	10.16	4.32	0.011 [y]
Oxygen level	1	16.64	7.08	0.001 [y]
Egg size x Oxygen level	1	0.07	0.03	0.989 [y]
Residual	41	2.35 [x]		

b) Univariate tests:

Source		Survival			Length			Weight		
	df	MS	Pseudo-F	p (perm)	MS	Pseudo-F	p (perm)	MS	Pseudo-F	p (perm)
Year	1	3.89	4.12	0.049 [y]	5.01	7.03	0.011 [y]	0.01	0.02	0.892 [y]
Egg size	1	0.32	0.34	0.565 [y]	2.23	3.13	0.081 [y]	7.61	11.00	0.002 [y]
Oxygen level	1	0.26	0.28	0.595 [y]	8.40	11.78	0.001 [y]	7.98	11.54	0.002 [y]
Egg size x Oxygen level	1	0.06	0.07	0.802 [y]	0.00	0.00	0.997 [y]	0.01	0.01	0.920 [y]
Residual	41	0.95 [x]			0.71 [x]			0.69 [x]		

726
727
728 [y] - Term mean squares tested against the pooled mean squares of residuals and remaining non-
729 significant interaction terms.
730 [x] – Mean square of pooled residuals and remaining non-significant interaction terms.
731
732

733 Table 2 – Permutational MANOVA and ANOVA analyses of the effects of egg size treatment
734 (small vs. large), oxygen treatment (100% vs. 40%), year (2007, 2008), day (1, 9 and 18) and
735 male ID (nested within egg size, oxygen level and year) on *Syngnathus. typhle* a) overall and
736 b) separately for ventilation rates, proportion of time spent swimming and proportion of time
737 spent in the upper half of the aquarium. N = 41.
738

a) Multivariate test:

Source	df	MS	Pseudo-F	p (perm)
Egg size	1	9.29	3.14	0.037 [y]
Oxygen level	1	44.21	14.94	< 0.001 [y]
Year	1	7.87	2.66	0.054 [y]
Day	2	14.61	2.71	0.177
Year x Day	2	5.38	2.68	0.032 [y]
Male	36	2.94	1.47	0.019 [y]
Residual	79	2.01 [x]		

b) Univariate tests:

Source	Ventilation rate				Proportion of time swimming				Proportion of time in upper part of tank			
	df	MS	Pseudo-F	p (perm)	df	MS	Pseudo-F	p (perm)	df	MS	Pseudo-F	p (perm)
Egg size	1	0.00	0.00	0.977 [y]	1	7.08	7.49	0.010 [y]	1	2.21	2.43	0.132 [y]
Oxygen level	1	43.45	39.39	< 0.001 [y]	1	0.23	0.24	0.631 [y]	1	0.52	0.57	0.450 [y]
Year	1	1.96	1.78	0.201 [y]	1	5.87	6.21	0.017 [y]	1	0.04	0.04	0.843 [y]
Day	2	4.41	13.61	0.105	2	0.43	0.25	0.851	2	9.77	2.91	0.239
Year x Day	2	0.32	1.13	0.324 [y]	2	1.70	1.84	0.164 [y]	2	3.36	4.21	0.021 [y]
Male	36	1.10	3.85	< 0.001 [y]	36	0.95	1.02	0.452 [y]	36	0.91	1.14	0.317 [y]
Residual	79	0.29 [x]			79	0.93 [x]			79	0.80 [x]		

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741 [y] - Term mean square was tested against pooled mean square of residuals and all other
742 interactions.
743 [x] – Mean square of pooled residuals and non-significant interaction terms.
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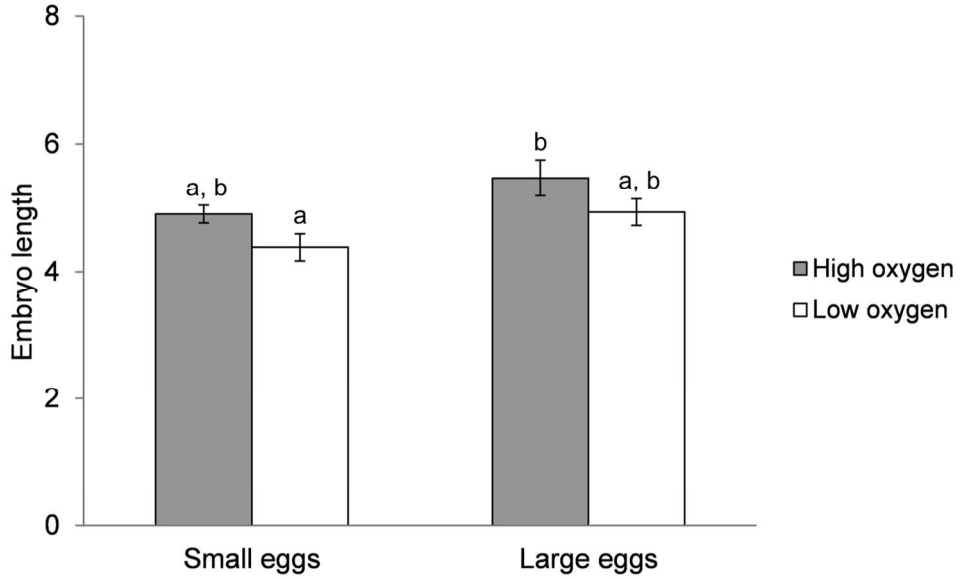
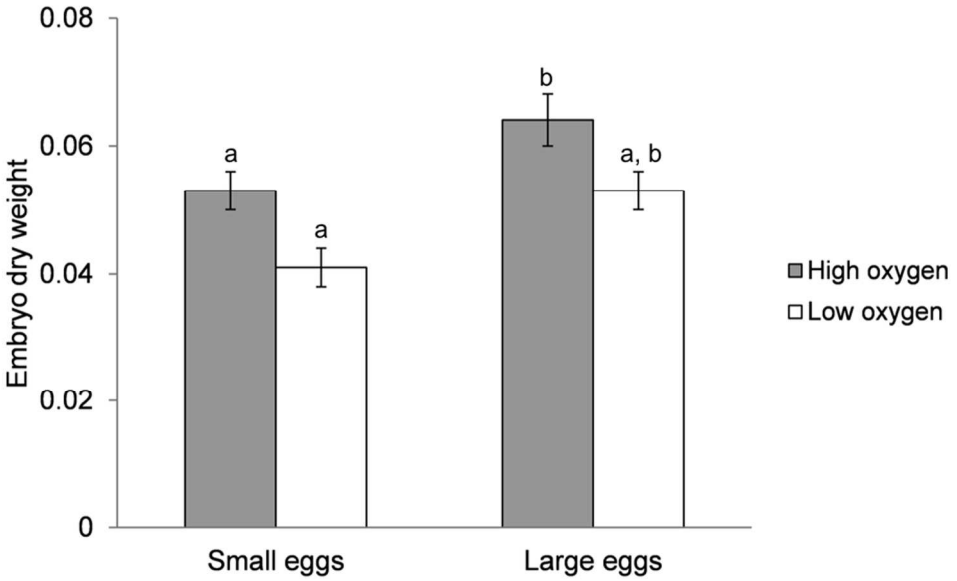
Figure 1: a) Average embryo length (mm) and b) average embryo dry weight (mg) of *Syngnathus typhle* males that received small or large eggs and were kept either in high (100%) or low (40%) oxygen conditions for a brooding period of 18 days. Significant differences are displayed with different letters, based on LSD post-hoc tests following 1-way ANOVA, using an oxygen level-egg size composite treatment factor with four levels.

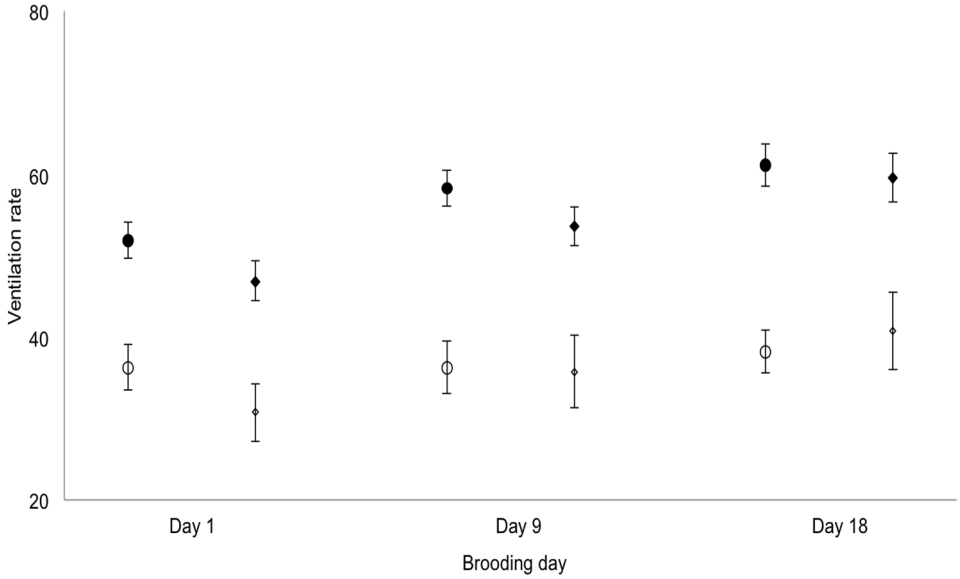
Figure 2: Average (mean ± SE, ventilations per minute) ventilation rates of *Syngnathus typhle* males brooding embryos from large (circles) or small (diamonds) eggs, kept in high (open symbols) or low (filled symbols) oxygen conditions. Ventilation rates were recorded three times during the experiment, on days 1, 9 and 18.



952x571mm (96 x 96 DPI)



952x571mm (96 x 96 DPI)



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